Abstract. Vitamin D metabolism was studied in the 'Hannover Pig', a strain which suffers from pseudo vitamin D-deficiency rickets, type I. Animals of this strain are known to be devoid of renal 25-hydroxyvitamin D₃-1α-hydroxylase and -24-hydroxylase activities. Pigs with florid rickets and hypocalcaemia were treated with single im injections of 0.25 to 1.25 mg of vitamin D₃, doses that have been shown in previous studies to be effective in producing transient healing of rachitic symptoms. The levels of vitamin D₃ and its most relevant physiological metabolites in plasma were estimated at intervals before and after this vitamin D₃ treatment. Vitamin D₃ rose from 14.8 ± 8.1 to 364 ± 190 nmol/l (mean ± sd) 2 to 3 days post injectionem, 25-hydroxyvitamin D₃ from 131.0 ± 46.2 to 1068 ± 160 nmol/l within 7 days post injectionem. The 1α,25-dihydroxyvitamin D₃ concentration in plasma was elevated from 73.9 ± 25.0 to 281 ± 168 pmol/l 2 to 3 days post injectionem and declined continually from that time. 24R,25-dihydroxyvitamin D₃ and 25S,26-dihydroxyvitamin D₃ levels after treatment showed different responses in different animals being either elevated or unchanged. Clinical healing of the pigs with these doses of vitamin D₃ was attributed to the transient rise of 1α,25-dihydroxyvitamin D₃ in plasma. It was assumed that 1α,25-dihydroxyvitamin D₃ synthesis takes place under these circumstances in extrarenal tissues.

In 1962 a pig strain was described (Plonait 1962) which developed clinical symptoms of rickets at weaning, despite a normal dietary supply with vitamin D. Subsequent studies revealed that the disease was caused by an inherited defect and was transferred to offspring by an autosomal recessive gene. Clinical, haematological and endocrinological studies showed that homozygote animals developed symptoms similar to classic vitamin D-deficiency rickets. This included hypocalcaemia, increased activity of alkaline phosphatase, secondary hyperparathyroidism, impaired mineralization, and accelerated mobilization of bone mineral (Harmeyer et al. 1982). It was demonstrated in an in vitro system using renal cortex homogenates that no 1α-hydroxylase activity was present in the kidney even in presence of high concentrations of 25-hydroxyvitamin D₃ (25-OHD₃) (Fox et al. 1985; Winkler et al. 1986). These and other parameters of vitamin D metabolism investigated in the rachitic pig strain were similar to those found in humans suffering from pseudo vitamin D deficiency rickets type I (Fanconi & Prader 1969).

Animals of the rachitic pedigree usually die at an age of 6 to 12 weeks when remaining untreated. They can, however, effectively be treated with pharmacological doses of vitamin D₃ that are 20 to 100 times above the physiological daily requirement of approximately 8 to 10 µg. As (1α,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) production in non-pregnant animals and humans is assumed to take place only in the kidneys (Gray et al. 1971; Fraser & Kodicek 1970; Shultz et al. 1983), it is not clear why pharmacological doses of vitamin D₃ can heal rachitic symptoms in pigs and humans lacking renal 1α-hydroxylase activity.

This finding also leads to the question why large doses of vitamin D₃ or 25-OHD₃ can effectively

Vitamin D₃ metabolism in a pig strain with pseudo vitamin D-deficiency rickets, type I

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heal symptoms of bone disease in patients with chronic renal failure. In such patients a normalization of plasma calcium, an increase in 25-OHD₃ and calcitonin, and a decrease of plasma PTH were observed after treatment with 1 to 2 mg of vitamin D₂ daily (Fröhling et al. 1982). In other studies treatment of patients with renal failure with 100 μg of 25-OHD₃ daily cause a rise in plasma 1,25-(OH)₂D₃, an effect which was not observed in anephric patients (Zerweck et al. 1982). The 1,25-(OH)₂D₃ levels in untreated anephric patients varied, however, from zero to 20.4 ng/l plasma (Jongen et al. 1982).

It is attempted with this study to measure the time course of vitamin D₃ and vitamin D₃ metabolites in plasma of pigs which suffer from pseudo vitamin D-deficiency rickets, type I after treatment of the animals with pharmacological and effective doses of vitamin D₃.

Material and Methods

Experimental animals

Protocol 1. Seven rachitic litter mates of the 'Hannover Pig', 4.0 to 7.9 kg body weight, were used for the experiments. All animals were cross breeds between the German Landrace and the Göttinger Miniature Pig to which the rachitic trait was transferred. An amount of 10 ml of blood was collected by puncture of the internal jugular vein 3 to 4 days before and at 3- to 4-day intervals after treatment. The animals were treated with a single im injection of 1.25 mg of vitamin D₃.

Protocol 2. Blood samples were collected from 3 rachitic piglets, 1.7 to 5.8 kg body weight, before and 2 to 3 days after an im injection of 0.25–0.5 mg of vitamin D₃. This dose of vitamin D₃ represented the lowest possible dose that was clinically effective. Plasma was prepared from heparinized blood samples by centrifugation at 3500 x g and was stored at −20°C. Plasma Ca and the activity of alkaline phosphatase were estimated according to procedures provided by Boehringer, (Mannheim, FRG).

Protocol 3. Eight heterozygote piglets 5 to 8 weeks old served as normal controls. Quantities of 10 ml blood were obtained by venous puncture. Blood sample preparation was as in protocol 1 and 2.

Analysis of vitamin D₃ and of vitamin D₃ metabolites

Vitamin D₃ and its metabolites were estimated in plasma by a modified procedure of Lambert et al. (1981) and Horst et al. (1981a). Aliquots of 1 to 4 ml of plasma were extracted with methanol/methylene chloride. The extract was fractionated on Lipidex 5000 (United Technologies, Frankfurt, FRG) into three fractions which were brought to dryness and subsequently isolated on high-pressure liquid chromatography (HPLC) columns at different conditions. 1,25-(OH)₂D₃ was finally estimated in the HPLC eluate using a radioimmunoassay. For recovery estimates known amounts of ³H-labelled vitamin D₃ metabolites were added before extraction of plasma.

Extraction procedure

One volume of plasma was added to three volumes of a mixture of methanol/methylene chloride (2 + 1, v/v) and was mixed for 2 min. One more volume of methylene chloride was then added and mixed for another 2 min. The mixture was centrifugated for 10 min at 12 000 x g. The lower solvent phase was collected with a pasteur pipette. Two more volumes of methylene chloride were added to the supernatant phase thoroughly mixed and recentrifuged. The lower solvent phase was again collected and combined with the first solvent fraction.

Fractionation of the extract

The lipid extract was brought to dryness, redissolved in 300 μl n-hexane/chloroform (96 + 4, v/v) and applied to 70 x 10 mm columns filled with Lipidex 5000. For fractionation we used a 3-step n-hexane/chloroform gradient, consisting of: a) 22 ml 96% n-hexane, b) 18 ml 85% n-hexane, and c) 24 ml 45% n-hexane. The flow rate was 2.3 ml/min. The three fractions contained: a) vitamin D₃, b) 25-OHD₃, c) the double hydroxylated metabolites 1,25-(OH)₂D₃, 24R,25-dihydroxyvitamin D₃ (24,25-(OH)₂D₃), and 25S,26-dihydroxyvitamin D₃ (25,26-(OH)₂D₃), respectively. The fractions were dried in vacuo and used for further HPLC chromatography.

High pressure liquid chromatography

Vitamin D₃ and its metabolites were separated and identified at normal phase conditions using 4.6 x 250 mm Zorbax-Sil columns (Du Pont, Bad Nauheim, FRG) and n-hexane/isopropanol as eluent, flow rate 1.5 ml/min. The conditions were for fraction a: 0.5% isopropanol, isocratic (retention time of vitamin D₃ 24 min), for fraction b: 2.4% isopropanol, isocratic (retention time of 25-OHD₃ 23 min), for fraction c: 8% isopropanol, isocratic 9 min, gradient of isopropanol 8 to 15% during 34 min (retention time of 24,25-(OH)₂D₃ was 12 min, of 25,26-(OH)₂D₃ 23 min, and of 1,25-(OH)₂D₃ 32 min). Separation of vitamin D₃, 25-OHD₃ and 24,25-(OH)₂D₃ from contaminating substances was often not complete. In these cases the eluate fractions of the HPLC chromatogram containing vitamin D₃ or vitamin D₃ metabolites were again brought to dryness and applied to a reversed phase Zorbax-OBS column (Du Pont, Bad Nauheim, FRG). The samples were re-chromatographed with methanol/water; vitamin D₃
with 4% water, isocratic (retention time 18 min), 25-OHD$_3$ with 12% water, isocratic, (retention time 14 min), 24,25-(OH)$_2$D$_3$ with 20% water, isocratic, (retention time 15 min). The flow rate was also 1.5 ml/min.

The individual metabolites were identified by retention times and radioactivity of labelled markers which were added prior to extraction. All compounds except 1,25-(OH)$_2$D$_3$ were quantified photometrically at 265 nm. The lowest limit of detection of the UV photometric system was 3.75 pmol/l for both vitamin D$_3$ and for the vitamin D$_3$ metabolites. This resulted in a sensitivity of about 1.75 nmol/l plasma of each individual metabolite when 4 ml of plasma were used with an average recovery of 50%.

**Radioimmunoassay of 1,25-(OH)$_2$D$_3$**

An antibody against 1,25-(OH)$_2$D$_3$ from sheep was used. Preparation and specificity of the antibody has been described (Clemens et al. 1979). The 1,25-(OH)$_2$D$_3$ peak after HPLC separation was identified by radioactivity in 100 µl of 1.0 ml fractions. The combined fractions were brought to dryness and redissolved in 120 µl of ethanol. Recovery was estimated from 2 x 20 µl aliquots. The radioimmunoassay was carried out in triplicate using three 20 µl aliquots. Each aliquot was mixed with 10 µl of [26,27-methyl-3H]1,25-(OH)$_2$D$_3$, (specific activity 6.67 TBq/mmol) in ethanol solution containing 66 Bq, 250 µl of antibody in phosphate buffer (67 mmol/l + 1% BSA + 0.5% NaN$_3$, pH 7.4). The mixture was incubated in the dark at 4°C for 15 to 24 h. Fifty µl of activated charcoal (3 g Norit GSX + 0.3 g dextran T20 or T70 + 100 ml phosphate buffer without BSA) was then added, mixed and incubated for 15 min. The mixture was centrifuged for 15 min at 2500 × g and removed from the centrifuge exactly 10 min later.

Two hundred µl of supernatant was used for liquid scintillation counting. Unspecific binding was estimated from a mixture which contained the same compounds except sample and antibody. Maximum binding at zero substrate concentration was about 35% of the radioactive ligand present. 1,25-(OH)$_2$D$_3$ concentrations were calculated using a nine point standard curve with 1,25-(OH)$_2$D$_3$ concentrations between 4 and 125 pg, estimated in triplicates. The curve was smoothed by spline function (Reinsch 1971). Recoveries were for vitamin D$_3$ 48.9%, for 25-OHD$_3$ 54.2%, for 24,25-(OH)$_2$D$_3$ 59.9%, and for 1,25-(OH)$_2$D$_3$ 55.5%. Since radioactive labelled 25,26-(OH)$_2$D$_3$ was not available, the recovery of 25,26-(OH)$_2$D$_3$ was calculated from the mean recoveries obtained for 1,25-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$. All radioactive labelled metabolites used in this procedure were rechromatographed before use.

Solvents were purchased from Merck (Darmstadt, FRG). Solvents used for extraction had the quality grade ‘for residue analysis’; solvents used for HPLC chromatography were of ‘chromatographical grade’. N-hexane was redistilled before use. Vitamin D$_3$ was from Serva, (Heidelberg, FRG). Vitamin D$_3$ metabolites were kindly provided by Hoffmann-La Roche, (Basel, Switzerland). The radioactive labelled metabolites were from Amersham Buchler, (Braunschweig, FRG).

| Table 1. Concentrations of vitamin D$_3$ and its most relevant metabolites (means ± SD) in plasma of rachitic piglets (N = 13), and normal non-rachitic control animals (N = 8). |
|-----------------------------------------------|-----------------|-----------------|----------|
| Vitamin D$_3$ (nmol/l) | Control piglets | Rachitic piglets | n.s. |
| 25-OHD$_3$ (nmol/l) | 63.2 ± 55.3 | 109.6 ± 58.6 | n.s. |
| 24,25-(OH)$_2$D$_3$ (nmol/l) | 21.6 ± 7.4 | 10.7 ± 4.7 | P < 0.01 |
| 25,26-(OH)$_2$D$_3$ (nmol/l) | 15.4 ± 10.9 | 9.7 ± 8.8 | n.s. |
| 1,25-(OH)$_2$D$_3$ (pmol/l) | 169.2 ± 64.0 | 71.9 ± 25.9 | P < 0.01 |

**Results**

**Concentrations of vitamin D$_3$ and vitamin D$_3$ metabolites in plasma**

Concentrations of vitamin D$_3$ and vitamin D$_3$ metabolites in plasma from rachitic untreated piglets and from heterozygote controls are listed in Table 1. No significant differences were found for vitamin D$_3$ between rachitic piglets and normal controls. However, both 1,25-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$ were significantly lower in rachitic piglets compared with heterozygote controls.

**Vitamin D$_3$ metabolites of rachitic piglets treated with vitamin D$_3$**

The rachitic condition of piglets was verified by hypocalcaemia and by the presence of clinical symptoms of rickets such as bone deformities, reduced food intake, muscle weakness, and fati-
I

Vitamin D3 -2 to 0 2 4 6 8 10 12 14 16 18 20 22

Days after treatment
• p<0.05 compared to control

Fig. 1.
Plasma concentrations of calcium and of the activity of alkaline phosphatase in rachitic piglets before and during 21 days after treatment with pharmacological doses of vitamin D3. The first value represents control level before treatment (mean ± SD, N = 7 piglets).

Peak concentrations of Ca were seen about one week after treatment. From this time plasma calcium started to decline gradually. Treatment with vitamin D3 resulted in transient healing of clinical signs. The animals showed greater mobility, probably owing to relief of pain from bone and joints.

Time courses of vitamin D3 and vitamin D3 metabolites before and after treatment are shown in Fig. 2 and 3.

Vitamin D3 increased from physiological concentrations of 13 nmol/l to 390 nmol/l with a peak between the 2nd and 4th day. Its concentration dropped rapidly thereafter and reached near physiological levels 7 to 10 days later. 25-OHD3 continued to increase during a period of 7 days reaching peak concentrations of more than 1000 nmol/l. From that time a gradual decline was seen during the following 2 weeks. Three weeks after
Plasma concentrations of 24,25-(OH)₂D₃, 25,26-(OH)₂D₃ and 1,25-(OH)₂D₃ in rachitic piglets before treatment and during 21 days after intramuscular injections of pharmacological doses of vitamin D₃. The first value represents control levels before treatment. Vertical bars indicate means ± SEM; horizontal bars indicate sampling intervals; numbers = number of piglets.

Fig. 3.

**Discussion**

**Pre-treatment concentrations of vitamin D₃ and vitamin D₃ metabolites**

The concentrations of vitamin D₃ and its metabolites in plasma confirm earlier preliminary estimates (Harmeyer et al. 1982; Kaune & Harmeyer...
and OHD3 significantly (OH)2D3 (Fox 1985) and agree with results of other laboratories (Fox et al. 1985). The data show that the 1,25-(OH)2D3 level is significantly lower in rachitic animals than in normal controls but is still significantly above zero. 24,25-(OH)2D3 was also significantly lower than in the controls, whereas 25-OHD3 levels were higher. Plasma 1,25-(OH)2D3 and 24,25-(OH)2D3 appear to contrast with results of in vitro estimates of renal cortex 1α-hydroxylase- and 24-hydroxylase activities from rachitic pigs (Fox et al. 1985; Winkler et al. 1986) where no activities of these enzymes could be detected. The activity of 1-hydroxylase was easily measurable in kidney preparations from normal control animals (Winkler et al. 1986). The sensitivity of the cited assay allowed detection of 5 to 10% of the 1α-hydroxylase activities present in normal pigs. Thus, there is little reason to believe that the two compounds present in plasma of rachitic animals and in this study designated 1,25-(OH)2D3 and 24,25-(OH)2D3 are of renal origin.

Analysis of vitamin D3 and vitamin D3 metabolites

Identification of 1,25-(OH)2D3 and 24,25-(OH)2D3 seemed to be sufficiently specific in our study, unless yet unknown compounds exist which comigrate with these metabolites and cross-react with the antibody. Although no mass spectroscopy could be carried out, identification of 1,25-(OH)2D3 rested upon several specific criteria such as the reasonable specificity of the antibody and comigration of 1,25-(OH)2D3 in the HPLC system with the labelled marker. Indeed the antibody used in this study is not specific for 1,25-(OH)2D3 (Clemens et al. 1979), but chromatographic separation from other cross-reacting metabolites like 25-OHD3, 24,25-(OH)2D3 and 25,26-(OH)2D3 was sufficient. For further verification, 2.7 ml fractions of the HPLC eluate were assayed in the radioimmunoassay system. The profile of the immunologically detectable 1,25-(OH)2D3 in the 2.7 ml fractions coincided with the radioactivity profile of the fractionated peak (Fig. 4).

Identification of 24,25-(OH)2D3 was undertaken after comigration of the substance with the labelled marker on normal phase and on reversed phase HPLC columns after separation from 25-OHD3-26,23-lactone. By the described procedure, vitamin D3 and vitamin D2 analogues of the various metabolites except 1,25-(OH)2D3 were also separated, but the quantities of D2 metabolites were negligible. Investigations by Horst et al. (1981a) have also shown that vitamin D3 does not play a significant role in pigs. So we assume that most, if not all, of the 1,25-(OH)2D was 1,25-(OH)2D3.

Control of vitamin D3 metabolism after vitamin D3 treatment

The increase in vitamin D3 and 25-OHD3 in plasma after treatment shows that the vitamin D3 was administered in an absorbable form and that the hepatic 25-hydroxylase is not altered under the conditions of pseudo vitamin D-deficiency rickets, type I.

Marked increases in vitamin D3 and 25-OHD3 in plasma have also been observed in normal
nephrectomized pigs after im injections of 125 mg of vitamin D₃ into 5- to 6-weeks old pigs (Littledike & Horst 1982; Horst et al. 1981b). Owing to the more than 100-fold higher dose used by those authors, vitamin D₃ reached 3- to 7-fold higher plasma concentrations, but 25-OHD₃ concentrations increased little more than twice the level present in our experiments.

The rise of 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ in our pigs demonstrates their capability tosynthesize these metabolites in the absence of the renal enzymes. The presence of 24-hydroxylase activities have been described in various organs other than the kidneys such as bone, cartilage, the intestine, placenta, and skin. 1α-hydroxylase activities have been described in the placenta of rats and humans, in bone of humans and chicks, in foetal intestine of chicks, and human melanoma cells or sarcomatous macrophages (for references see: Mason 1985). But these extrarenal 1α-hydroxylases are assumed to play no significant role in healthy non pregnant animals or humans (Mason 1985). This agrees with findings in experiments with nephrectomized rats which failed to produce significant amounts of 1,25-(OH)₂D₃ even when treated with pharmacological doses of 25-OHD₃ (Boyle et al. 1972; Shultz et al. 1983). It contrast, however, with findings obtained from nephrectomized pigs which produced significant quantities of a vitamin D metabolite comigrating on HPLC with 1,25-(OH)₂D₃ (Littledike & Horst 1982). The same study also showed a continuing increase in plasma 24,25-(OH)₂D₃ and 25,26-(OH)₂D₃ following injection of pharmacological amounts of vitamin D₃. The authors speculate that extrarenal synthesis of 24,25-(OH)₂D₃ and 25,26-(OH)₂D₃ takes place when plasma 25-OHD₃ concentration exceeds 1000 nmol/l. In our experiments this threshold concentration of 25-OHD₃ was reached only for a few days post injectionem, which perhaps is the reason for the variability of the response of 24,25-(OH)₂D₃ and 25,26-(OH)₂D₃.

Another possibility for the healing effect of large doses of vitamin D₃ in renal failure was suggested by Kumar et al. (1981). They found significant amounts of 5,6-trans-25-hydroxyvitamin D₃ (5,6-trans-25OHD₃) in rats given 5,6-cis-vitamin D₃ orally, which binds more efficiently to intestinal cytosol-binding protein than 25-OHD₃. In our experiments we have not measured 5,6-trans-25OHD₃, but preliminary experiments had shown that treatment with 40 µg of 5,6-trans-25OHD₃ daily was not effective in healing rickets in our pigs. Therefore we assume, that a possible formation of 5,6-trans-25OHD₃ cannot explain our results.

Another interesting point is the observation that 1,25-(OH)₂D₃ in plasma began to decline already when the 25-OHD₃ level still rose. Although it was difficult exactly to define the time of the maximum 1,25-(OH)₂D₃ concentration there is clear evidence that 1,25-(OH)₂D₃ markedly declined (7 days post injectionem) before plasma 25-OHD₃ reached its maximum concentrations. On the basis of our studies we cannot tell whether and how extrarenal 1,25-(OH)₂D₃ production is controlled after the administration of pharmacological doses of vitamin D₃. The plasma Ca concentration reached its maximum a few days after the peaking of plasma 1,25-(OH)₂D₃ and closely paralleled the time course of 1,25-(OH)₂D₃.

Littledike & Horst (1982) reported the same for nephrectomized pigs treated with 20 to 125 mg of vitamin D₃. It remains unclear whether PTH plays any significant role in 1,25-(OH)₂D₃ regulation under this condition. The low 1,25-(OH)₂D₃ levels which are present in plasma of untreated animals with marked secondary hyperparathyroidism show that only a low rate of 1,25-(OH)₂D₃ production is maintained in the presence of 25-OHD₃ concentrations nearly twice the physiological level.

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